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BIOLOGICAL BULLETIN

MODIFICATION OF RESPONSE IN AMŒBA.

S. O. MAST AND L. C. PUSCH.

FROM THE ZOÖLOGICAL LABORATORY OF THE JOHNS HOPKINS UNIVERSITY.

Gibbs and Dellinger ('08) and Schaeffer ('17) maintain that Amœba has the power of selecting food. The former hold that the kind of food preferred changes under various conditions, and they suggest that changes in the kind of food selected is the result of a process of learning based upon the method of "trial and error." One of us (Mast '10) in preceding observations found that when the tip of a pseudopod of an amœba comes in contact with a highly illuminated region in the field it stops; then others are sent out in succession in the same general direction, each one stopping when it comes in contact with the light, until suddenly one is sent out in a markedly different direction, avoiding the illuminated region altogether. It is maintained that this abrupt change in the direction of the formation of the pseudopods, constituting a profound modification in the response to a given stimulus, is dependent upon the preceding experience of the individual, *i.e.* repeated contact with the strong light.

The specimens used in the following experiments were obtained from the cultures in the laboratory, most of which had been stocked with material from a stream near the campus. They were of the proteus type. Each amœba was isolated on a glass slide in a drop of its culture-fluid under a cover-glass, the edges of which were sealed with vaseline. Each amœba was adapted to darkness before using.

The observations were made in the dark room. An area of intense light was obtained by focusing on the slide under the microscope, by means of the reflector and an Abbe condenser, an image of a portion of the luminous filament of an incandescent Mazda lamp placed in a light-tight box. The rays of the filament passed through a very narrow slit in the box. The image had

clearly defined edges and furnished a band of intense light across the field. The slide was adjusted so that the amœba was within a distance equal to approximately half its length from the light with the pseudopod or pseudopods progressing towards it. If the pseudopod did not stop when it came in contact with the light the amœba was discarded. If it stopped the behavior of the amœba was observed until it definitely moved away from the light, and the number of pseudopods which made contact with it was recorded. After this the light was shut off, and the amœba was left in darkness until the next trial was made. In this way each individual was given twenty-seven trials.

The interval between trials was approximately three minutes except in the following as recorded in Table I: individual *B*, trials 16–17 and 23–24, 24 hours; individual *D*, trials 15–16, 22 hours; individual *E*, trials 8–9 18 hours, 16–17, 48 hours, and 21–22, 24 hours; individual *F*, trials 8–9, 50 hours, and 20–21, 20 hours; individual *G*, trials 16–17, 16 hours, and 24–25, 3 hours. During these intervals the amœbæ were kept on their slides in darkness undisturbed.

The behavior of a typical individual is illustrated in Fig. 1 by sketches representing all of the trials in the series. By referring to this figure it will be seen that while during the first part of the series there was something of a persistence on the part of the amœba to attempt to proceed in its original direction—notably 7 attempts in trial 4—there was but little in the latter part of the series—a total of only 5 attempts during the last 14 trials.

The results obtained in all of the tests are presented in Table I. This table shows that the number of pseudopods which came in contact with the light before the direction was changed decreased in general as the number of trials increased in each series, that there was a similar decrease in the total contacts of all amœbæ for corresponding trials, and also in the totals of consecutive groups of three trials. Amœba *B* made 10 contacts with the light in the first three trials; with trial 4 a general decrease begins. A total of 20 pseudopods made contact in the first 14 trials of the series, and a total of 9 in the last 13. In the series of amœba *D* there were 5 contacts in the first 6 trials but none at all in the remaining 21 trials. Amœba *E* made 21 contacts in the first 14 trials—11 of these were in the first 4—and 5 in the last 13

FIG. 1. A series of diagrammatic sketches illustrating the behavior of Amœba *E* for twenty-seven trials. The straight lines of equal length represent a band of light. The outlines of the amœba describe its position in relation to the light at the time given below in each individual sketch. The arrows show the direction of protoplasmic streaming.

trials. Amœba *F* made no contacts until trial 8; between trials 8 and 15 there were 8 contacts, while in the remaining 12 trials there was but one contact. There was one contact in the first trial of Amœba *G*, then none until the 7th, when a reversal began and persisted very generally through the 17th, by which time 23 contacts had been made; in the remaining ten trials there were 8 contacts.

TABLE I.

RESPONSE OF AMŒBA TO CONTACT WITH A HIGHLY ILLUMINATED REGION IN THE FIELD.

Designation of Trials.	Number of Pseudopoda that Came in Contact with Light in Each Trial.					Total Contacts for All Individuals in Each Trial.	*Total Contacts for All Individuals in Groups of Three Consecutive Trials.
	B.	*D.*	*E.*	*F.*	*G.*		
1	4	0	0	0	1	5	
2	3	0	3	0	0	6	
3	3	3	1	0	0	7	18
4	0	0	7	0	0	7	
5	3	1	0	0	0	4	
6	0	1	0	0	0	1	13
7	1	0	2	0	1	4	
8	2	0	0	2	4	8	
9	0	0	2	1	2	5	17
10	0	0	4	0	1	5	
11	3	0	2	2	2	9	
12	0	0	0	0	0	0	14
13	0	0	0	1	2	3	
14	1	0	0	0	4	5	
15	0	0	1	2	1	4	12
16	1	0	1	0	1	3	
17	0	0	0	0	5	5	
18	0	0	0	0	0	0	8
19	0	0	1	0	0	1	
20	0	0	0	0	1	1	
21	2	0	2	0	0	4	6
22	0	0	0	1	0	1	
23	1	0	0	0	3	4	
24	0	0	0	0	3	3	8
25	4	0	0	0	1	5	
26	1	0	0	0	0	1	
27	0	0	0	0	0	0	6

The totals by groups of three consecutive trials fluctuate between 18 for the first group, 12 for the middle and 6 for the last. In other words there is a modification of response in the sense that the number of attempts to continue in the original direction after meeting the light decreases in general as the number of trials of the series increases.

It will be remembered that each series was interrupted irregularly by longer intervals of time, usually about a day or longer. Despite this there is a general constancy of the tendency throughout each series. This seems to indicate ability to retain for some time the modification of response.

These experiments were repeated 18 months after the preceding portion of the paper was completed. Two individuals were used and a series of 18 readings approximately 3 minutes apart were obtained with each. The total number of contacts made by these two individuals in groups of three consecutive trials beginning with the first follows: 20, 9, 10, 6, 7, 4. The results obtained in these observations are consequently in harmony with those obtained in the preceding observations.

SUMMARY.

When Amœba repeatedly comes in contact with a band of intense light the number of attempts to continue in the original direction decreases as the number of trials increases.

This indicates that there is some change in Amœba that is analogous to what is called "learning" in the higher animals.

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AN EXPERIMENTAL STUDY AND A PHYSIOLOGICAL INTERPRETATION OF EXOGASTRULATION AND RELATED MODIFICATIONS IN ECHINODERM EMBRYOS.

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INTRODUCTORY.

In a study of the means of altering and controlling the course of development in echinids by electrolytes the writer had occasion to note the action of lithium chloride in effecting remarkable form changes in the early ontogeny of sand-dollars and sea-urchins. An attempt was then made to analyze further these effects and the similar ones so extensively studied by Herbst and others, and to provide for the wealth of facts obtained some more consistent and satisfactory explanation than has been offered.

Application to these cases of the susceptibility and differential inhibition methods of Child has supplied what seems a secure and reasonable basis for interpreting such puzzling teratological forms as exogastrulæ, "holentoblastulæ," etc. For I believe that the first impression of an exquisitely specific action of the Li' ion is not sustained by a closer examination of the facts, and that "lithium larvæ" fall logically into the series of abnormal forms producible quite at will by other means.

The work on *Echinarachnius*, *Arbacia*, and *Asterias* was carried out at Woods Hole in the summer of 1916 and that on *Strongylocentrotus* and *Orthasterias* at Friday Harbor in 1917. For materials and facilities at these stations the author is indebted to Dr. F. R. Lillie and Dr. W. C. Frye, and for many suggestions, discussions, and even for generous contribution of experimental data special thanks are extended to Dr. C. M. Child.

EXPERIMENTAL.

Physiological Axial Gradients.—In scores of species of plants and animals of all degrees of complexity of organization a gradient

of metabolic activity and of other associated properties and conditions (CO_2 production, O_2 consumption, electrical potential, vital staining capacity, etc.) has been demonstrated by the help of many different methods (Child, '15*b* '20). Of these methods that of determining the relative susceptibility of various levels along the structural axis is most readily applicable in embryos, and has been found a generally reliable, if somewhat gross, indicator of the presence and nature of the physiological gradients, the significance of which for development has been emphasized by this writer.

Methods.—As applied to ontogenetic studies there are really *two interrelated susceptibility methods:* (*a*) direct determination of susceptibility of different parts of the egg or embryo; and (*b*) differential inhibition, differential acclimation and differential recovery in development.

(*a*) Agents in concentrations determined to be lethal within a few hours commonly cause death and disorganization first of the parts of the egg or embryo with most rapid metabolic activity and then later attack in succession in the same way those portions with progressively lower rates of metabolism—the susceptibility paralleling roughly the metabolic gradient (direct susceptibility). In lower concentrations which kill after a day or more the order of susceptibility was found to be reversed, since regions with more active metabolism acclimate more rapidly and completely (indirect susceptibility). For these purposes a vast number of lethal agents have been employed with generally concordant results, but the more toxic agents, such as cyanides, anesthetics, salts of heavy metals, basic dyes, etc., usually give best results; and with some of these tests were made on the direct susceptibility of the lithium-modified as compared with normal embryos (p. 73).

(*b*) When developmental stages are treated with the same agents in partially lethal concentrations or sublethal concentrations permitting acclimation or tolerance, or are returned to the normal medium after temporary exposure, a differential effect on development results. That is, the levels of highest metabolic rate in any gradient are more retarded or inhibited, or in case of acclimation or recovery, acclimate or recover more rapidly or more completely than levels of lower rate. In differential in-

hibition then the levels of lower rate may be relatively accelerated and in differential acclimation or recovery the levels of higher rate may be relatively accelerated after the primary inhibition. Direct differential acceleration is also possible under proper conditions. These differential effects of external agents in relation to the physiological gradients provide means of controlling to a greater or less extent the course of development and the sizes, proportions and relations of parts under given conditions (Child, '16, '17; Bellamy, '19).

In short, the established gradient of the egg or embryo, being physiological, is not necessarily permanently fixed and unalterable, but may be radically modified, obliterated entirely (as in *Asterias* embryos by N/1000 HCl in sea water), or even entirely reversed in direction, as we shall show for the lithium larvæ (Fig. 13).

The ordinary procedure in the differential inhibition method was as follows: Eggs of freshly collected animals were removed to a bowl, fertilized, and then placed during early cleavages in small quantities in liter Erlenmeyer flasks of sea water to which the agent used had been added. The flasks were loosely covered but not aërated. Each day the solution was renewed and embryos examined and notes and sketches taken recording the course of their development as compared with the controls kept in pure sea water. In most experiments some of the embryos were also removed after various times of exposure to the salt solutions to determine the possibility and nature of the steps of their recovery.

The Lithium Modifications.—Best results with lithium are contingent upon recognition of the prominent rôles of several factors; especially concentration of the agent, duration of exposure, and stage of development at time of exposure. In higher concentrations development is strongly inhibited and incomplete. If the concentration is too low, or if exposure is too brief, begins too late or is terminated at too early a stage, the effect is weak and development approaches by all transitions towards the normal. Herbst observed ('95*a*, ch. 3) that typical effects are not produced by temporary treatment merely up to fertilization, cleavage or young blastula stages; but well developed blastulæ treated with rather concentrated solutions and then returned to

sea water do show some of the lithium modifications much obscured by recovery. In general it may be said that maximal effects may be secured by sea water solutions of about N/60 to N/200 LiCl (according to the species of the embryo used) acting for a sensitive determining period from cleavage continuously through late blastula stages.

Echinarachnius parma.—This sand-dollar provides perhaps the most responsive material yet found for the study of lithium effects, which are here, if anything more diverse and extreme than in sea-urchins and far more so than in the star fishes used.

The action of lithium solutions producing the characteristic lithium effects is such as to retard or inhibit development, the degree of inhibition (and loss of power of recovery) varying directly with concentration. LiCl, 2 H_2O, N/60 or more, in sea water stops cleavage early; and even at N/80 only a few individuals reach the blastula stage. These survivors are either partial blastulæ or very small, immobile, thick-walled stereo-blastulæ, whose remarkably small segmentation cavity is packed so full of precociously formed opaque mesenchyme that much of the latter is forcibly extruded from the basal pole; these types, though not "sickly," die shortly without gastrulation. In N/100 death occurs most commonly in late cleavage, but one finds, inactive at the bottom of the container, a few stereo-blastulæ, which die as such, or, rarely, after assuming the constriction form described below.

In N/125 LiCl a considerable minority of the eggs become opaque mesenchyme-filled blastulæ, the larger of which, especially in more dilute (N/150) LiCl, often show a wide zone of thickening of the basal pole and after some delay begin the elongation preluding gastrulation; but the entoderm then evaginates after the fashion of that in lithium exogastrulæ. There is much variation in the diameter of the ring (usually basal) where the ectodermal (gastrula wall) and entodermal (archenteron) portions of the embryo join, but this ring is always enlarged—often so much so that it moves toward, to, or even past the equator of the blastula (Fig. 1, *n-q*); in the extreme cases the exogastrulation process appears to be merely a constriction between the animal and vegetal poles, forming two vesicles, one consisting

of the gastrula wall and the other of the gut entoderm. These two components may be of very different relative size (Fig. 2)

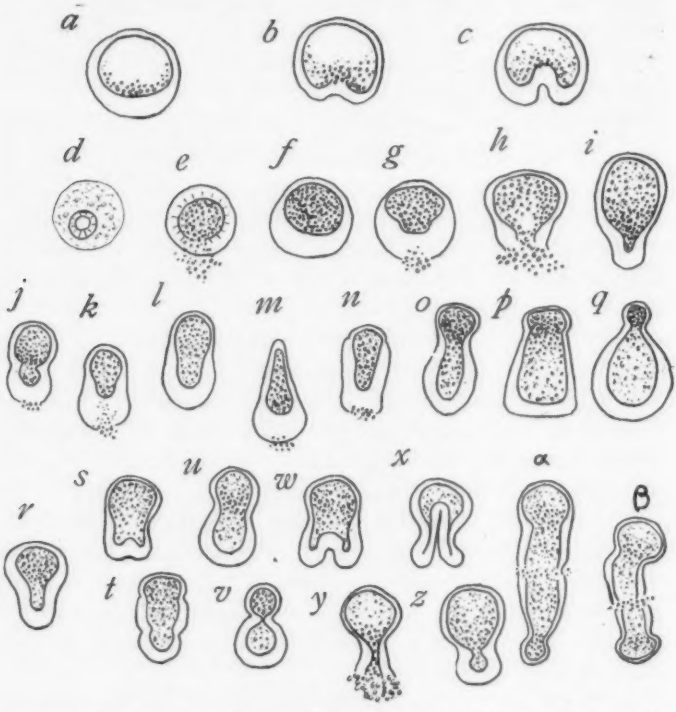

FIG. 1. Blastulæ and early gastrulæ of the Atlantic sand-dollar, *Echinarachnius parma*. *a–c*, normal embryos; others, typical embryos as modified by lithium. In the lithium series note: the overdevelopment and frequent basal extrusion of the mesenchyme; the various degrees of enlargement of the sharply defined, thick-walled endodermal portion; and the occasional occurrence (*s*, *w*, *x*) of partial endogastrulation. *α* and *β* are double exogastrulæ, two joined terminally by their basal ends.

as might be expected from the mode of their formation. Sometimes the ectodermal portion exceeds the entodermal, often they are more or less equal, but in a great many the entoderm is clearly much the larger. In the last mentioned cases the ectodermal component becomes less and less, and approaches the vanishing point—a little knot of cells or nothing at all!—while the archenteron undergoes a compensatory and reciprocal hy-

pertrophy, until in a few individuals by its progressive enlargement the whole gastrula is entodermal (*i.e.* a "holentoblastula," better called for its physiological equivalent a holentogastrula).

There is never any real difficulty in distinguishing ectoderm and entoderm in these forms. In every species there are characteristic differences in pigmentation, in thickness of walls, in structure of the cells, in cilia, in size of cells, in frequency of

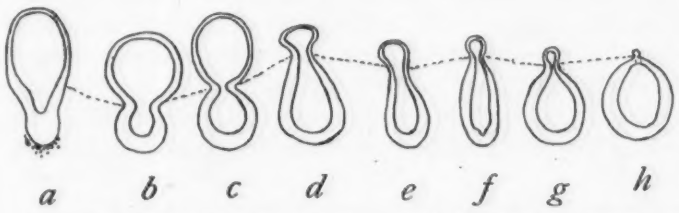

FIG. 2. Series of sand-dollar embryos (constriction forms) developed 27 hours in M/160 LiCl. Ectoderm (above) separated at dashed line from endoderm. Mesenchyme packing the blastocoels omitted. The ectodermal (thin-walled) component, larger in *a–c*, is reduced in size in *d–f*, and nearly obliterated in *g*, which is practically a holentogastrula.

nuclei, etc. From a study of the lineage of cells from the pigmented and nonpigmented zones of the early egg it may be stated quite positively that entoderm grows at the expense of ectoderm, some of which becomes converted into entoderm (and possibly also into mesenchyme).

Left in the solution many of these constriction forms show early death and gradual disintegration, sometimes of the apical ectoderm, or of both extremes, but usually of the entodermal component from its free end towards the attached part. Returned to sea water the more resistant ectodermal portion may recover and linger on awhile as a partial form, a holectogastrula as it were. This type may arise also in another way: if the constricted gastrulæ are removed early from the lithium and allowed to recover in sea water ectoderm and entoderm tend to separate from each other, especially when the two components are of nearly equal size (Fig. 3, *v*). It would seem that equality and duality are inconsistent with development of one individuality!

N/160 is close to the optimal concentration for producing the most striking lithium effects in this species. Retardation of

development is less general and more localized and differential. The eggs produce practically 100 per cent. modified blastulæ, which are smaller and thicker walled than their controls; the ectoderm does not thin out for some time even though the blastocœl is almost invariably solidly filled with mesenchyme. Such overproduction of mesenchyme seems to lead to a basal extrusion and loss of excess proliferated primary and secondary mesenchymal cells from the basal pole of the blastula, which is commonly

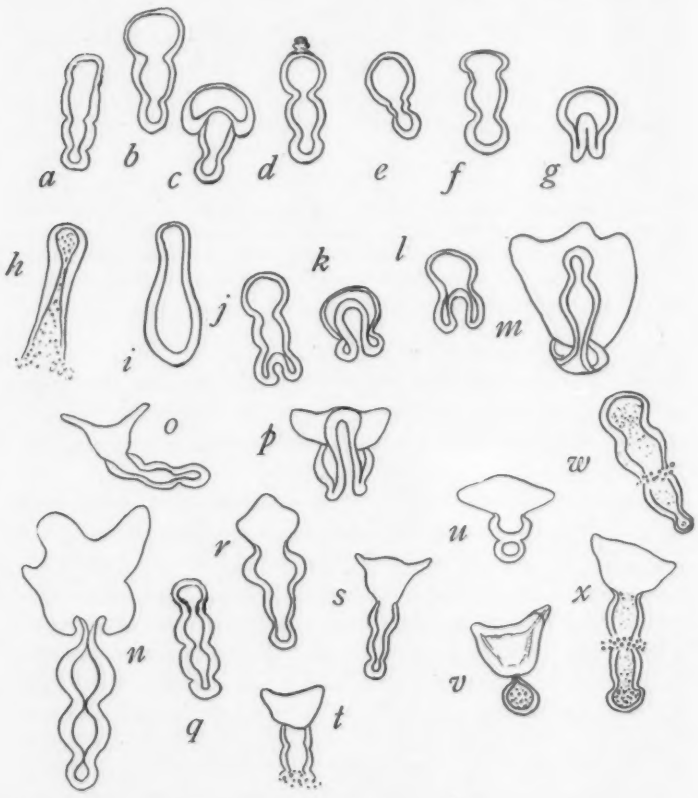

FIG. 3. Commonest types of sand-dollar gastrulæ and larvæ from lithium cultures. Show the wide range of lithium alterations. (See also other examples in the originals of Fig. 12). *a–i*, from M/160 and *j–x* from M/200 LiCl, returned at about 2 days to sea water. Mesenchyme omitted in most.

marked by the "cell rosette" of Herbst, composed sometimes of isolated cells and sometimes of loose masses, as if the blastula wall had actually burst at its weakest point through distention of its cavity. Shortly the entoderm begins to evert, all stages of exogastrulation are present (Fig. 3), and soon the culture consists almost entirely of exogastrulæ with only a few forms approaching normal endogastrulation. And here again one finds the whole gamut of types with regard to the relative size of the ectodermal and entodermal portions. The ectoderm shows all degrees of reduction down to complete obliteration in the large, relatively clear, holentogastrulæ, whose walls and contents exhibit all the distinguishing features of the archenteron. Nothing is more evident than that the entoderm grows here at the expense of the ectoderm.

At 33–36 hours or more those types in which both components are present may show further differentiation: the archenteron shows constrictions separating it into two, or its usual three divisions. This tri-partite gut is safely homologized with fore-, mid-, and hind-gut, with the fore-gut of course, situated at the most posterior free end of the embryo. The relative size of these three divisions shows a most interesting series of variations (Fig. 4). The total gut is greatly increased in bulk over the

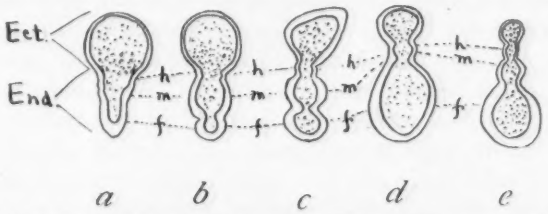

FIG. 4. Selected series of types from sand-dollar cultures developed in LiCl, M/150 to M/200. Showing, *a–e*, the wide range of reductions in the ectodermal portion (*ect.*); and the parallel changes of proportion of the endoderm (*end.*) in its three divisions: hind-gut (*h*); mid-gut (*m*); and fore-gut (*f*).

normal and could not conceivably be enclosed in the cavity of the ectoderm; and in this increase the fore-gut takes the greatest share, the mid-gut next, and the hind-gut (attached to the ectoderm) the least. There is an obvious and significant parallel in this process to the relative increase of the entoderm as a whole at the expense of reduction of the ectoderm.

Herbst demonstrated quite conclusively ('95 *a*, *e.g.* Figs. 17, 18, 22) that the thick entodermal layer of the lithium larvæ and of the intermediate types, is rich in nuclei and in the number of its smaller cells in proportion to the size of the part. The ectoderm, on the other hand, gives every evidence of retarded activity: at first very thick, it afterward becomes thin and stretched but is never composed of more than a single cell layer (except when and where the ciliary bands appear), and this layer is peculiarly sparse in nuclei and is composed of a few extraordinarily large cells, both in comparison with the normal ectoderm and with the entoderm of lithium larvæ.

The tip of the fore-gut often shows some disintegration, and the resulting cellular debris and associated mesenchymal cells are evidently somewhat sticky, for one finds frequently that two exogastrulæ fuse, telosynaptically as it were, by the ends of their guts (not laterally as in Herbst's cases). These adhere closely and more or less permanently to form "*fused twins*" (Fig. 3, *w*, *x*) whose parts, of varying relative size, have an interesting history of inhibition and reciprocal overgrowth, separation, etc., that might be followed further with profit.

Meanwhile differentiation has progressed somewhat in the *ectodermal* component also; especially if this is fairly large, has not been too strongly inhibited, and is then allowed to recover partially in sea water. Notable deviations from the normal development are seen in the marked reduction and generally radial disposition of apical structures. The mouth usually fails to form, but when it does it is practically terminal. The ciliary bands, when formed, appear as an apical ciliated tuft, plate, or small transverse ring. Apical outgrowths are common (Fig. 3, *d*). By the third day skeletal spicules have often formed in the mesenchyme, which is collected, not in two lateral masses near the blastopore, but, rather, crowded far forward into the apical end of the cœlome; these spicules stimulate the production of the pluteus arms, which are uncommonly close together and narrow angled from the marked reduction of the oral lobe and also from the tendency of the "anal" arms to be formed very near the oral region. Herbst also noted ('95, *a*, Figs. 32–36) that lithium larvæ successfully recovering in sea water continue their overproduction of mesenchyme and skeletal structures and

lay down an excess number of skeletal spicules (3, 4, or 5—the total final number in ordinary development—or even 7, in *Sphærechinus*) and the pluteus arms are increased in number and displaced accordingly and had besides an increased number of radii (4 or 5 instead of 3).

N/200 LiCl slows down development noticeably, but the blastulæ are of nearly normal size and less crowded with mesenchyme, which is extruded from the posterior end in only about half of the embryos. When gastrulation occurs there are found about 60–70 per cent. entogastrulæ and only 30–40 per cent. exogastrulæ and intermediate forms. The blastulæ recovering in sea water quickly resume active movements and become gastrulæ differing from the lithium larvæ in the slightly thinner ectoderm and entoderm; in the incomplete or merely temporary (Fig. 3 *g*, *j-m*, *p*) evagination of the gut; in the relatively large

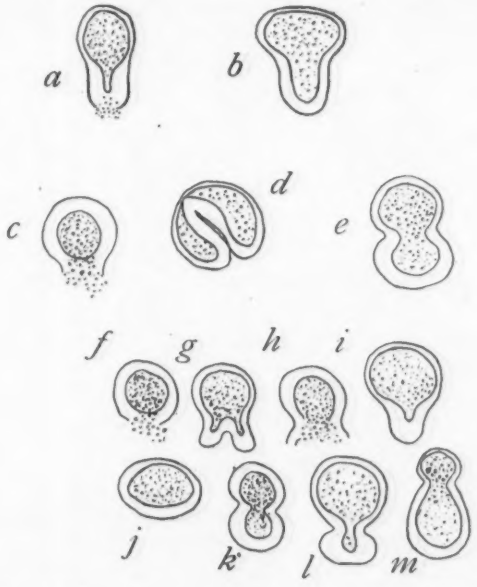

FIG. 5. Sand-dollar embryos as modified by $CuSO_4$, $HgCl_2$, KNC, and dilute sea-water. *a*, an early exogastrula after 1 day in $CuSO_4$, M/1,250,000; *b*, after 2 days from $HgCl_2$, 3M/10,000,000; *c–e*, from diluted (80 per cent.) sea-water; *f-m*, from KNC M/750,000.

gastrular wall and more fully divided gut; and in their larger oral region and occasional belated evagination of the stomadeum.

N/250 LiCl yields mostly ordinary forms, but little retarded, and only 5–10 per cent. exogastrulæ. In still lower concentrations development quickly approaches that of the controls.

Other agents, such as KNC, $CuSO_4$, $HgCl_2$, HCl, $CaCl_2$, NaCl, and conditions, such as diluted or staling water, used in appropriate concentrations and intensities, were found to cause the same sort of general inhibition of development as LiCl, stopping entogenetic changes at cleavage, blastula, or gastrula stages; all may be employed to show the relatively greater inhibitory effect on apical and ectodermal parts; many brought the blastula up to a stage (Fig. 5) precisely simulating late Lithium blastulæ; and a few produced a small percentage of imperfect exogastrulæ, as in Fig. 5.

Arbacia punctulata and Strongylocentrotus franciscana.—In these sea-urchins a range of types of abnormality was produced

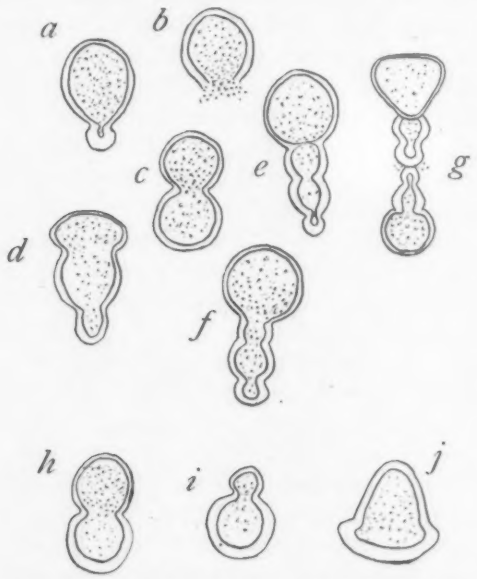

FIG. 6. Exogastrulæ, Constriction Forms, and Double Embryo of *Arbacia*, produced by LiCl and NaCl. *a–g* from LiCl, M/80 to M/100; *h–j*, from NaCl, M/4 to M/8.

by lithium treatment, deviating from the normal in the same ways and essentially to the same degrees as do the sand-dollar lithium larvæ. The concentrations required are rather higher, the most numerous and typical lithium effects being induced in the Atlantic species by about N/80 to N/100 LiCl, and in the Pacific form by N/100 or less.

Several hundred sketches of teratological types in these urchins (the very common types seen in Figs. 6, 7) conform so

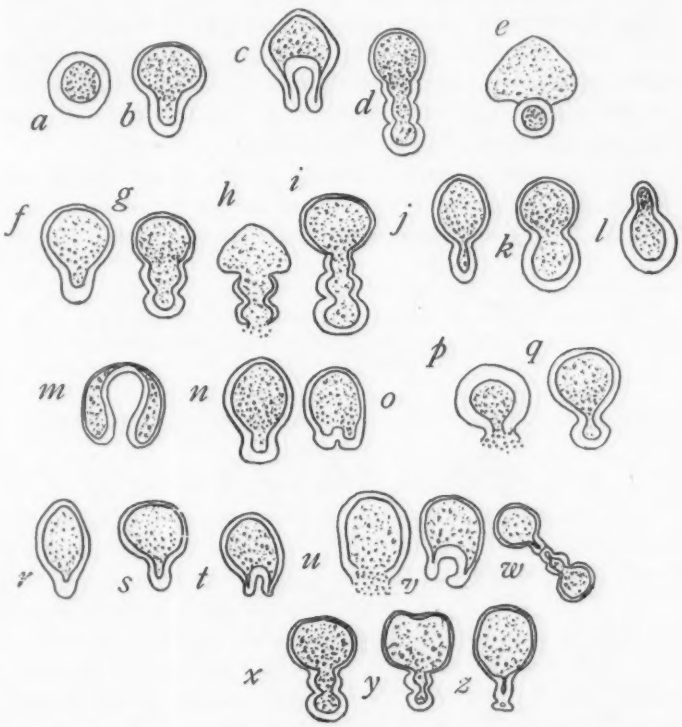

FIG. 7. Modified *Strongylocentrotus* Embryos. *a–e*, from LiCl, M/200; *f–l*, from LiCl, M/150; *m–o*, from NaCl, M/16; *p–q*, from KNC, M/250,000; *r–z*, from $CaCl_2$, M/160 to M/200.

closely to the sand dollar forms just described and to the modifications illustrated and described in such great detail by Herbst ('92, '95*a*, '95*b*) for *Echinus microtuberculatus*, *Sphærechinus*

granularis and *Strongylocentrotus lividus*, that further description would hardly aid in our present purpose of analyzing and interpreting the lithium effect.

It is important to mention the production of practically typical lithium embryos without the aid of lithium. Herbst found a good substutute for the lithium salts in Na butyrate etc., and Driesch ('95) obtained a high percentage of exogastrulæ from development at 30° C. for nearly a day preceding gastrulation. So it is not entirely surprising to find that in $CaCl_2$, N/80 to N/160, often the majority of *Strongylocentrotus* eggs become characteristic exogastrulæ, etc. (and many of these fused twins) practically indistinguishable in type from those in Figs. 1–3. Quite similar exogastrulæ are produced in *Arbacia* by NaCl, N/4 to N/8, and in *Strongylocentrotus* by N/8 to N/20 NaCl.

Asterias forbesii and Orthasterias sp.—Starfishes are by no means as responsive as the urchins and sand-dollar to the lithium action. Though all characteristic stages of general inhibition are

FIG. 8. Experimental Alterations of *Asterias*, *a–e* induced by LiCl, M/100 to M/160; *m–p*, induced by 75 per cent. sea-water; and *q*, by KNC, M/25,000

readily obtained by the lithium salts and other agents, and apical inhibition is evident in the characteristic reduction or entire suppression of the preoral ciliary field, etc., yet the endogastrulation process is not so easily interfered with.

But partial and occasionally fairly typical exogastrulæ occur with great regularity in *Asterias* cultures in N/100 to N/200 LiCl solutions in KNC, in diluted sea water, etc. (Fig. 8).

In *Orthasterias* N/160 LiCl sometimes induces exogastrulation or marked change of proportions of the germ layers, and not uncommonly in later development the stomadeal pouch is turned outward and forward from the mouth by a process apparently similar to exogastrulation (Fig. 9.).

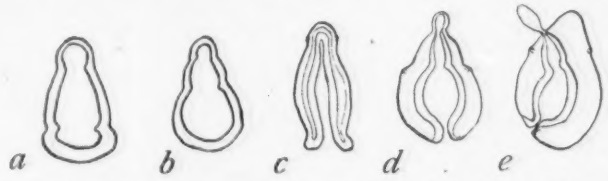

FIG. 9. Alterations of *Orthasterias* embryos in M/160 LiCl. *d–e*, showing stomadeal eversion; *c*, with mixed endo- and exo-gastrulation; *a–b*, with reduced ectoderm and greatly enlarged endoderm, particularly in the fore-gut. Note the position of the ciliary bands in *c–e* and in FIG. 8, *c*.

Comparison of Susceptibility gradients of Normal and Lithium Embryos.

1. *The Normal Embryos.*—The susceptibility relations in the early ontogeny of *Asterias* and *Arbacia* have been described by Child ('15*b*; '16*a*); these observations have been confirmed and extended to include the sand-dollar (data by Dr. Child and the writer) and the Pacific coast forms.

According to the direct susceptibility method (p. 61) the fertilized or dividing eggs, blastulæ, gastrulæ and larvæ were placed in lethal solutions of KNC (M/100 to M/1000), $HgCl_2$ (M/50000 or less), $CuSO_4$, concentrated neutral red or methylene blue, and to a few other salts, etc. and the course and relative times of death of parts noted by the criteria of possibility of recovery in return to sea water and of disorganization of cell structure. Disorganization is often made more visible either by a brief return to sea water, or by previous or simultaneous staining in

dilute neutral red since the stained tissue decolorizes sharply at the death point. Since general susceptibility increases steadily from fertilization to gastrulation the concentration of the agents was correspondingly decreased in the later stages.

The order of susceptibility in the succeeding stages of development is shown in Fig. 10. Disorganization, manifested by droplet

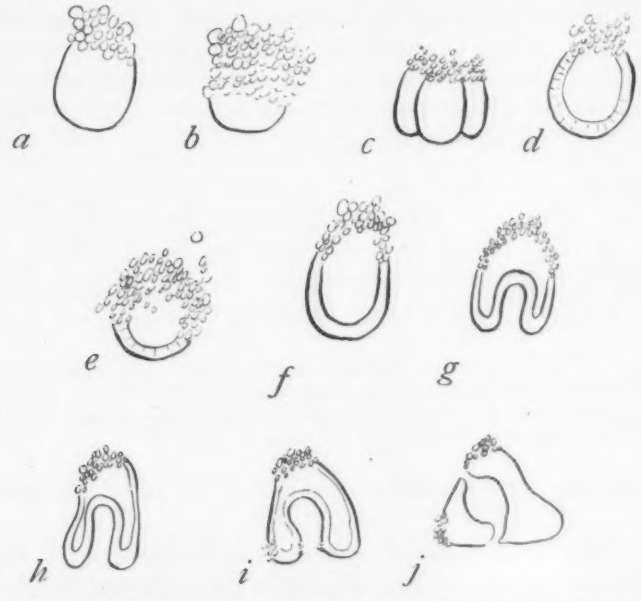

FIG. 10. Disintegration of normal sand-dollar eggs and embryos, showing relative susceptibility of parts. Mesenchyme omitted. *a–b*, eggs; *c*, 4-celled; *d–f*, blastulæ; *g–j*, gastrulæ and early larvæ. When the polar axis becomes recognizable disintegration proceeds from animal to vegetal pole (*i.e.* is *apico-basal*).

formation, cell separation, roughening of contour, visible disintegration, and decolorization, according to the particular agent used, begins at one point of the surface of the egg, or at one pole of the blastomeres in the 2- or 4-cell stage, or at one pole of the cleavage mass or early blastula, and then extends gradually over adjacent parts to reach last the opposite surface or pole.

As soon as the blastula differentiates to show the basal thickening and begins its elongation, one may orient the embryo in

its chief apico-basal axis at a glance. Here the manifestations of death are first visible at the animal pole or apex and progress gradually and steadily towards the basal region which is reached last, often hours after the opposite end had been affected. The apparent reverse in some slow-acting solutions was obviously due entirely to the basal extrusion of the mesenchyme.

The same gradient persists into the gastrula stage, where the apex shows the greatest susceptibility, while the invaginated entoderm is so much more resistant (except probably for a zone around the blastopore) that it usually survives in M/100 KNC an hour or more after the ectoderm of the entire gastrular wall has disintegrated. By returning non-disintegrating gastrulæ to sea water after an appropriate exposure the ensuing disintegration may be checked midway in its course and recovery larvæ are obtained showing absence or partial loss of apical (or even more basal) ectoderm and more or less complete survival of the entodermal parts which are accordingly large in proportion to the whole. As in the case of *Arbacia* "in the gastrula the apex of the conical body represents the apical end of the major axis, and the blastopore the basal end, while the entoderm represents a still more basal region of the egg and blastula, and the mesenchyme the most basal region" (Child, '16*b*, pp. 68–69).

Vital staining with neutral red and other basic dyes shows an apico-basal progression in blastulæ and early gastrulæ quite paralleling in all essentials the wave of advancing disintegration as described above.

The experimental embryologists have described the common

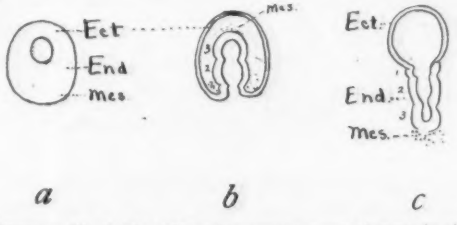

FIG. 11. Diagram illustrating the polar arrangement of parts in the egg (*a*), in the normal embryo (*b*), and in lithium embryos (*c*) of echinoderms. 3, 2, 1, fore-, mid-, and hind-gut. The lithium embryo is really an elongated and differentiated egg, with the germ-layers localized in the same order as were their antecedents in the egg.

plan of organization of the Echinid egg as such that the prospective significance of different levels of the egg along its main structural axis is as follows (see Przibram, '08): The animal pole half (diagram, Fig. 11. Ect.) furnishes the ectoderm and its differentiations; the subequatorial zone (End.) the gut and its derivatives; and the vegetal pole (Mes.) the primary mesenchyme and the larval skeleton. In Fig. 11 (*b*) and (*c*) these parts are homologized in the normal and the lithium-modified gastrulæ

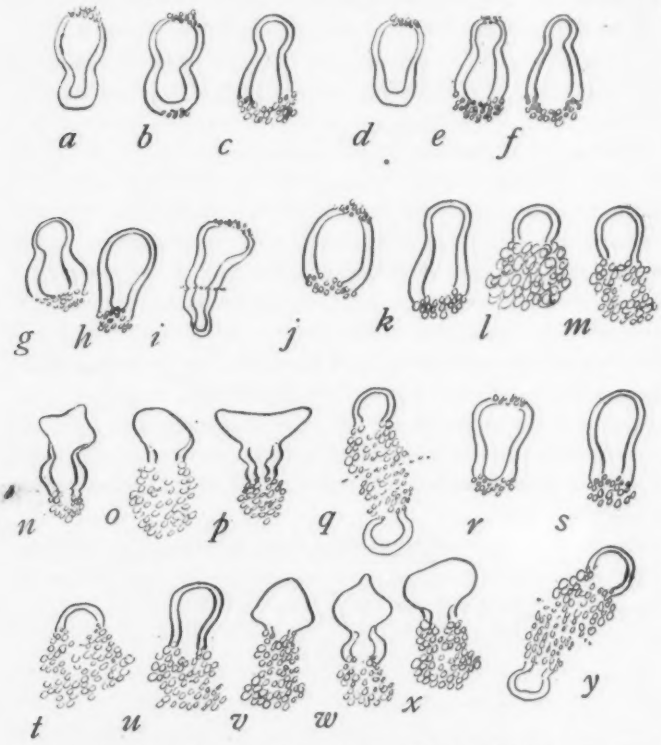

FIG. 12. Disintegration of typical lithium-modified sand-dollar embryos and larvæ. Mesenchyme omitted. *a–c* in neutral red; *d–i*, in $HgCl_2$ M/500000; *j–q*, in $HgCl_2$ plus Neutral Red; *r–y*, in KNC, M/1000; *i*, *q*, *y*, fused doubles. In the less modified forms (*a*, *b*, *d*, *j*, *r*) susceptibility is about equal apically and basally. In the more modified forms with large development of endoderm a distinct *basi-apical* disintegration gradient is always found. In general the larger the relative size of the endoderm and of the fore-gut the more distinct is this reversed gradient.

respectively. Obviously in the lithium-modified embryo the egg has merely elongated and differentiated without the usual infolding.

2. *Lithium Embryos.*—Tested with the same agents in precisely the same way as were the normal embryos the exogastrular and constriction forms (p. 63) showed new and different and characteristic susceptibility relations. General susceptibility is much less than in the normal, and the original apico-basal gradients of susceptibility, vital staining and decolorization are less marked, absent or reversed (Fig. 12).

Quite commonly the basal end of the entoderm has already begun disintegration in the LiCl solution itself, and much of the secondary mesenchyme may have been removed and the primary mesenchyme may have escaped from the blastocœl (Fig 12, *l*, *l*).

Newly formed lithium exogastrulæ and constricted forms removed intact to M/1000 KNC, M/500000 $HgCl_2$, or concentrated neutral red, disintegrate in the next few hours. In this process disintegration and decolorization after staining generally begins in the secondary mesenchyme-forming region of the archenteron.

Sometimes the two extreme ends disorganize at the same time, the apical ectoderm of the gastrula wall being as susceptibile as the basal entoderm of the archenteron. And in a few cases the ectoderm goes a little in advance of the opposite end. In these hour glass forms the susceptibility varies with the size of the components; the larger component being quite uniformly the more susceptible; and indeed it is apparently susceptible in direct proportion to its relative size, as the sketches suggest. The greater the reduction of the ectoderm the higher is its resistance.

Exogastrulæ removed from the lithium in a more differentiated state with bi- or tri-partite gut, etc., exhibit regularly the reversed order of susceptibility of parts (Fig. 12).

Discussion and Interpretation.

1. *Physiological Gradients in Echinid Embryos.*

It will be taken as highly probable that in the egg and blastula of the echinoderms so far studied the susceptibility declines from the apical animal pole region to the basal vegetal pole levels. In

the late blastula, gastrula and larva the susceptibility demonstrably declines sharply from ectodermal to entodermal and mesenchymal regions (p. 74). This is chiefly significant as indicating a corresponding gradient of metabolic activity—as a prerequisite to normal development—which is further evidenced and manifested by structural differences along the apico-basal axis (differences in cell size, cilia, pigment, vacuolization of protoplasm, etc.) and by functional differences in rate of developmental processes (growth, cell division, morphogenesis, and differentiation).

It is a familiar fact that teratogenic agents and conditions applied to whole eggs and embryos affect different regions of the developing organism differently and to a different degree. On the quantitative metabolic gradient conception regions of greatest susceptibility are subject to the greatest retardation in the developmental processes, while those of lowest susceptibility are correspondingly least inhibited, as is experimentally proven for *Arbacia* (Child, '16*a*), Annelids (Child, '17), the frog (Bellamy, '19), etc. Thus most teratological forms may be reproduced according to expectation and explained consistently.

Lithium effects also seem to be produced by a differential inhibition along a quantitative metabolic gradient. The facts relevant to this interpretation have been collected in the next section.

2. *Analysis of the Lithium Effects.*

Exogastrulation aside, lithium larvæ are characterized *morphologically* by the abnormal proportions of their parts, which involve all the germ layers in a manner we may summarize as follows:

(*a*) *Increase in amount and change in position of the mesenchyme and its derivatives:*

Excess mesenchyme of stereoblastulæ (p. 63).

Excess pigment cells (p. 63).

Excess skeletal structures, *e.g.*, spicules, arms, radii of arms, etc. (Herbst).

Conspicuous production of serous fluids and swelling of serous cavities in many annelid, fish, and amphibian embryos.

(*b*) *Hypertrophy of entodermal parts, especially basally:*

Relative and absolute increase of size of the gut in general, and of the fore-gut in particular (Figs. 2, 3, 4 etc.).

Thickness of entodermal wall (p. 68).

Richness of entoderm in nuclei (p. 68).

Small size of entodermal cells (Herbst; for frog, Bellamy '19).

(*c*) *Relative and Absolute Diminution of the Ectoderm, especially apically:*

Reduction of the ectodermal component of the hour glass forms (Figs. 2, 3).

Retardation of the apical region in differentiation (p. 69).

Retention of radial symmetry (p. 69).

Sparseness in nuclei (Herbst).

Large size of ectodermal cells (Herbst, Bellamy, Le Plat, '20, et al.).

Failure of ectoderm to grow down over the entoderm in the frog (Gurwitsch, '94, Morgan, '03*b*, Bellamy, '19, Le Plat, '20).

Since the main object of this paper is to deal with the lithium effect *physiologically*, it will profit to restate the morphological data just summarized in physiological terms:

Sufficiently strong concentrations of the Li ion will suppress development at any early or late stage of cleavage, of the blastula, etc. Slightly lower concentrations also retard development, but this retarding influence is most marked in the most susceptible region (ectoderm) of the embryo, distinctly less marked in the less susceptible entoderm, and least marked in the least susceptible mesenchyme. The last tissue undergoes a relatively rapid and excessive growth and abundant proliferation (in many ways resembling malignant tumor growth) to fill the blastocœle and lay down the anlage of an increased number of skeletal spicules (which at once push out the arms of the pluteus if recovery is permitted). The change of position of the mesenchyme may indicate a tendency (active or passive) to occupy spaces near cells of lowest metabolic activity.

The entoderm growth zone extends itself apically on to the gastrula wall (more and more as the gradient is levelled down or reversed) by virtue of an enhanced mitotic and growth activity, which is one of the most distinctive characters of a lithium embryo

and the feature by which the lithium effect may best be quantitatively estimated. The greater the lithium effect short of general inhibition, the greater the relative size of the entodermal as compared with ectodermal parts.

Meanwhile mitosis and growth have slowed down or utterly ceased in the ectodermal zone, whose development varies inversely with the development of the entoderm. In extreme cases the entodermal growth zone overspreads the whole blastula wall resorbing and incorporating and changing entirely the prospective significance of the apical region. This resorption doubtless proceeds in proportion to the relative rate of metabolic activity of the entoderm; just as in normal ontogeny the ectoderm feeds upon and limits the development of the gut, or as the nervous system of a starving animal maintains itself at the expense of parts of lower physiological activity. Here the ectoderm, usually the master germ layer, loses control and the more active entoderm gains the ascendency and may become the major part of the embryo. That the fore-gut of the evaginated archenteron also increases disproportionately may be attributed to the same process.

The inhibitory effect of lithium appears in the order: ectoderm > entoderm > mesenchyme; in the ectoderm itself: apical > basal; and in the entoderm: hind-gut > mid-gut > fore-gut (Fig. 13).

The most substantial direct proof of this reversal of the normal physiological gradient is found in the more or less complete reversal of the usual order of susceptibility; for in extreme cases the direct susceptibility was seen to be greatest in the fore-gut region and to decline towards the hind-gut and ectoderm (Fig. 13).

Judged then by the criteria of susceptibility, of developmental activity in mitosis, growth, rate of differentiation, etc., the metabolic gradient of the lithium larva is diminished, obliterated, or in the extreme case actually reversed (basi-apical). It is evident, however, that the reversal of the gradient occurs too slowly or too late to alter significantly the nature of the differentiated end products, for the respective germ layers bear recognizable similarity to these of the normal larva. Earlier exposure of the egg and a different technique of recognizing polarity would be neces-

sary to determine whether lithium can be made so far effective as to cause ectoderm to appear at the basal pole, etc.

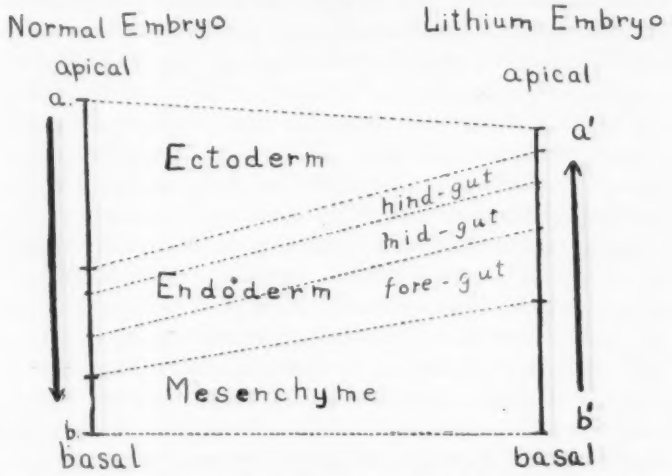

FIG. 13. Diagram showing the changed proportions of parts in a lithium embryo (right) as compared with a normal embryo (left), and the typical reversal of susceptibility (the arrows), *a–b* in normal, *b'–a'* in lithium embryos. The effect of a depressing concentration of lithium is to shift the embryo to the right. An accelerating agent would alter embryonic proportions in the opposite direction.

3. *Non-specificity of Lithium Effects.*

(*a*) *Changes of Proportions of Parts.*—A great variety of chemical agents and physical conditions have been employed in experimental embryology to cause general death and disintegration, general inhibition of development, differential (*i.e.*, localized) inhibition, and, in the minimal effective concentrations, differential acclimation effects. The striking fundamental similarity of teratological forms produced by diverse agents and conditions is too remarkable to be insignificant. There can be no one teratogenic substance or condition whose effect differs sharply in kind from others. Though each may have its own mode of attack, the end result is in general injurious and inhibitory or accelerative, according to the power of resistance of different levels along the metabolic gradient. The only specificity of lithium effect seems to lie in the higher regional differential susceptibility of many organisms to it; lithium has a particularly

marked capacity of inhibiting locally, retarding apical regions without appreciably hindering basal regions.

(*b*) *Exogastrulation*. In practice quite typical exogastrulæ are obtained in the sand dollar or in sea-urchins by all the lithium halides, sodium butyrate, KCl, etc. (Herbst), by $CaCl_2$, dilution or staling of sea water, etc. (this paper), by elevation of temperature to 30° C. during gastrulation (Driesch); and initial stages of exogastrulation by many other agents, as NaCl, $CuSO_4$, neutral red, etc. As regards exogastrulation specificity appears to reside chiefly in the biological material rather than in the external agent, for perhaps only in the pluteus-forming echinoderms is the lithium effect "typical"; in the starfishes, *Crepidula* (Conklin), and the sponge, *Oscarella* (Maas, '98) the effects are sufficiently marked and similar as to be recognizable; and in the annelids, ascaris, amphioxus, frogs (?) only negative results appear. Possibility of inducing exogastrulation depends, doubtless, on the specific constitution and organization of the egg, especially on the polar arrangement of the levels destined to form ectoderm, entoderm and mesenchyme. The physiological equivalents of exogastrulae may well appear quite different morphologically in different organisms.

4. *The Mode of Action of the Lithium.*

The problems of lithium action resolve into the causes of the two essential modifications: first, the disproportionate development of entoderm and mesenchyme; and second, exogastrulation. These are without doubt closely interrelated processes proceeding from a common fundamental cause.

Herbst admitted that he found himself in complete darkness on these matters but gave reasons for his opinion that the site of action of the salt must be in the egg itself rather than in its external membranes, and that Li' must produce a specific stimulating effect on vegetal pole and entodermal cells, whose capacity for taking up and retaining this ion he believed to be greatest ('95*a*, p. 185). Seeking to put the matter on a physico-chemical basis one of his students (Spek. '18) gave some evidence that if Li' did penetrate into certain (*e.g.*, entodermal) cells it would make for a greater imbibition of water by their colloids. Driesch ('95) believed that "the growth process of the blastula wall is

the vital fundamental process; the direction towards which the growth proceeds in exogastrulation is determined by the surroundings." It is suggestive that both these leading experimentalists appealed to some more or less differential growth process; but in neither case was the evidence more than meager, indirect, and isolated.

Attacked from the new viewpoint of differential inhibition, etc., lithium action is seen to be interpretable in a way not only entirely consistent and coördinate with a vast body of modern work in general and special physiology and of physiological form determination and teratomorphy, but also substantiated by considerable direct proof and many suggestive parallels (p. 78, and Child, '20). Since Li' in the concentrations actually necessary is certainly inhibitory and since the inhibitory effects are felt chiefly at the animal pole and in its differentiations and least in the mesenchyme and the entoderm, it is unlikely that the Li' acts so much directly on the basal regions; it may be said rather to *release* these regions from the control of the ectoderm by checking the (usually dominating) activity of the latter.

To determine the more proximate causes of the inhibitory effects will require much further detailed observation and experiment (for each separate agent) on sites and rates of penetration, water imbibition and withdrawal, alteration of surface tension and chemotaxis (Rhumbler, '02), precipitations, gelations, solutions, etc. Into whatever ultimate causal components resolved the end result remains, physiologically, a differential inhibition.

Exogastrulation is evidently a secondary manifestation of this differential action along a levelled or reversed metabolic gradient. Since the lithium-modified embryo is merely a lengthened and differentiated egg in which endogastrulation has been prevented (Fig. 11) exogastrulation might be considered even a direct result of the inhibition of these eggs (levelling the normal gradient necessary for endogastrulation).

But a mechanical factor seems to be involved. Expansion and overgrowth of the ectodermal epithelial layer is checked by the failure of the usual multiplication and enlargement of its cells, while growth and cell division of entoderm are less, and those of mesenchyme least retarded. Consequently the ectodermal cells

are few and large while the entodermal layer is rich in smaller nuclei. The mesenchyme cells proliferate in enormous excess and fill and overdistend the small blastocœl. Thus considerations of space and inner pressure alone render almost mechanically impossible the infolding of the entoderm which is, further, much longer, larger and thicker than usual, and is contiguous with the ectoderm by a vastly enlarged blastopore ring.

Certain other relevant facts should be noted: that the lumen of the exogastrular gut is really a part of the blastocœl or cœlome and is not the true gut cavity; that the constrictions of the differentiating gut are also therefore reversed in direction; that the stomadeum also often everts in starfish; that exo- and endogastrulation intergrade (there may be first one and then the other, or both at once). It is possible that the agent in obliterating and reversing the primary physiological gradient of the organism as a whole at the same time obliterates and reverses the functional polarity of the separate cells of the epithelial layers, so that insofar as polarity of cells aids in normal endogastrulation it might act in the opposite way in the lithium larvæ (by failure of the cells to widen and vacuolate basally, change their nuclear position, etc. cf. Gudernatsch, '13).

Tests of this interpretation of the lithium action may be made by an appropriate modification of the technique on other gradients (embryological, regenerating, motor). This agent might tend to reverse the polarity of regenerating pieces (*e.g.*, hydroid internodes, as an electric current does, Lund, '21), or of motor gradients (cilia, or muscular peristalsis), etc.

Summary and Conclusions.

1. Typical lithium larvæ may be produced from a high percentage of eggs of the sand-dollar, *Echinarachnius parma*, and of two sea-urchins, *Arbacia punctulata*, and *Strongylocentrotus franciscana*, by addition of LiCl to the sea water. These types approximate closely those obtained by Herbst in other urchins.

2. Morphological features common to and characteristic of these larvæ are (p. 78): (*a*) exogastrulation in many cases, and (*b*) always increase of mesenchyme and of entoderm and a compensating decrease of ectoderm. Further the most apical parts of the ectoderm and its differentiations are most decreased, and

the most basal parts of the gut are most increased, as the lithium alteration becomes maximal.

3. In the lithium larvæ the order of direct susceptibility of parts to quickly killing agents is essentially the reverse of that of the normal gastrula and larva: the gradient of susceptibility in the normal egg and blastula is apico-basal, and declines from the ectoderm to entoderm and mesenchyme in the gastrula and larva, while in the fully formed exogastrula the susceptibility of the ectoderm (gastrula wall) is least, that of the entodermal parts more, and that of the mesenchyme probably greatest of all. A similar reversal of the usual susceptibility is met with in parts of the differentiated gut of the lithium larva.

4. "Lithium larvæ" are not the specific product of the Li′ ion alone, but are readily obtained in $CaCl_2$, diluted or stale sea water, by Na butyrate (Herbst), by heat (Driesch), and in less typical form by many other salts, basic dyes, etc.

5. The specificity resides rather in the biological material than in the agent, but Li′ appears to possess in high degree the power of localized action: *i.e.*, its inhibition is markedly regional, slowing down or stopping development of the most apical parts, while hindering little or inappreciably development of the basal parts.

6. These induced, highly differential, rates of growth and differentiation lead directly or indirectly to exogastrulation in extreme cases; perhaps largely because of the mechanical impossibility of invaginating a greatly enlarged entoderm into an unusually small blastocœl, itself overcrowded by a precocious and excess proliferation of mesenchyme.

7. These changes in proportions and positions of parts in "lithium embryos" appear to follow as naturally and necessarily from the altered metabolic relations (more or less complete reversal of the physiological gradients), as the normal proportions and positions of parts follow from the normal metabolic relations.

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THE EXISTENCE OF A SHORT REPRODUCTIVE CYCLE IN *ANODONTA IMBECILLIS.*

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Fresh water mussels of North America have been classed by Sterki (1895) as summer or winter breeders. He notes that in winter breeders fertilization of ova occurs in the late summer, embryonic development is completed and the fully developed glochidia are carried through the winter in the maternal marsupia and discharged in the following spring. In the summer breeders fertilization occurs in the late spring or early summer and the fully formed glochidia are discharged "as a rule by the end of August."

Lefevre and Curtis (1912) prefer to designate these two classes as long and short term breeders. They include *Anodonta* in the former group, and although *A. imbecillis* is not listed in the data from which their conclusions are drawn, the statement is made that the breeding season is a generic character, "all species of a genus having essentially the same period of gravidity."

In a table compiled by Coker, Shira, Clark, and Howard (1919) gravid *Anodonta imbecillis* are listed for all months with the exception of April.

Anodonta imbecillis, although not important commercially, is of interest for two reasons; first, it is one of the two known species which carry the young in the marsupia during metamorphosis from glochidia to juvenile mussels, thus eliminating the parasitic stage on fishes which other species undergo, and, second, it is one of the few species which are hermaphroditic (Sterki, 1898).

During the summer of 1922 the writer attempted to work out a method of aparasitic mussel culture of commercially valuable mussels. One line of attacking this problem which seemed to offer promise of results was to simulate conditions pertaining in metamorphosis during the intra-marsupial life of aparasitic forms. Therefore a series of observations was begun on *Anodonta imbecillis* during the course of which successive examinations

were made at intervals of several days on more than one hundred individuals. This has furnished the following evidence for a shorter reproductive cycle than formerly reported for this species.

Material and Method.

Anodonta imbecillis is commonly called the "paper shell" because of the extreme thinness of the valves. Possibly correlated with this economy in shell formation, is its rapid rate of growth. It may begin to reproduce during the second year. The observations in this paper were made on two- and three-year-old mussels.

In this species the outer gills serve as marsupia. It is an easy matter to insert a capillary pipette between the valves, make a single puncture in the marsupium, and remove some of its contents with only slight injury to the mussel. This puncture heals rapidly. In order that the least possible modification of reproductive activity might be produced by this examination, the samples removed were small, containing only from 20 to 40 individuals. The puncture was made in a direction parallel to the long axis of the gill so that the sample removed contained young from several gill compartments.

Relatively slight disturbances may cause "abortion" of marsupial contents in some species. Lefevre and Curtis, however, note that they have never observed abortion in *Anodonta*. The thin shell of *A. imbecillis* renders the above described operation very simple as the valves may be opened for the insertion of the pipette with the thumb nails. This probably eliminates the possibility of abortion as a factor bearing upon the following evidence.

The examinations extended over a period of one and one half months from the middle of July to early in September.

Most of the mussels studied were taken from the main supply reservoir of the U. S. Fisheries Biological Station at Fairport, Iowa, which is supplied daily from the Mississippi River. A few preliminary observations were made on mussels taken from the river.

Throughout the observations they were kept in running water in experimental troughs supplied from a pond containing an abundance of pond life. As opportunity for considerable sedi-

mentation was afforded, this supply was much clearer than water from the river or reservoir. The fact that very successful yields were obtained in artificial mussel culture experiments carried on simultaneously under these conditions by Dr. A. D. Howard and B. J. Anson excludes any possible intervention of adverse environmental conditions.

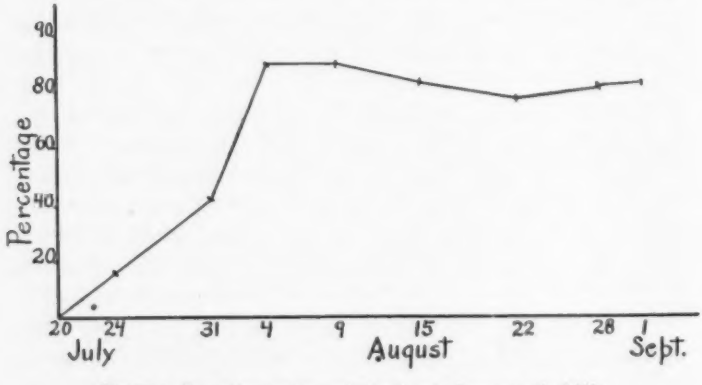
Observations.

Between July 15 and 20 about two dozen mussels were examined without finding a gravid individual. On July 24, 5 of 33 examined (15 per cent.) contained early embryonic stages. Nineteen of these 33 were observed repeatedly until September 1. On July 31, 8, including the five already noted (42 per cent.), contained embryos. On August 4, 17 or 89.5 per cent. were gravid. Larger numbers of this species were examined during August. From August 8 to September 1 the percentage of mussels bearing embryos, glochidia, or young in various stages of metamorphosis never dropped below 77.2 per cent. (see Table I. and Graph 1).

TABLE I.

Percentage of Mussels Bearing Embryos or Glochidia.

Date	7/15–20	7/24	8/4	8/9	8/15	8/22	8/28	9/1
Number examined	20–24	33	19	69	101	105	103	57
Percentage	0	15.1	89.5	89.9	83.2	77.2	80.6	82.5



Graph 1. Percentage of mussels bearing embryos or glochidia.

The conclusion drawn is that the reproductive season of *Anodonta imbecillis* in this location begins during the latter part of July.

As these observations were continued the various stages of embryonic development and glochidial metamorphosis were identified and data accumulated as to the time required for this transition. Eleven different stages were identified as follows:[1]

1. Early segmentation of the fertilized ovum.
2. Later segmentation.
3. Early differentiation of the single anlage of the valves which at this stage caps the segmenting cell mass.
4. Formation of a groove in this "cap" which divides it into two separate valve anlagen.
5. The developing valves lie on the segmenting cell mass "like continents in relief on the globe."
6. The two valves resemble a folded leaf which now completely encloses the future "soft parts" of the young larva.
7. The glochidial shape is attained but the larva is still colorless and transparent. The single glochidial adductor muscle can be seen to quiver or "flash" although the valves are closed.
8. The glochidia are now fully formed, have a rich yellow color, and a single adductor muscle. Many may be seen to snap vigorously if freed from the egg membranes in the process of removal from the marsupia. Those within the membranes have their valves opened. This stage is equivalent to that at which the glochidia of species dependent on parasitism on fishes for metamorphosis are extruded from the marsupia.
9. Mid-metamorphosis; rich yellow in color, no adductor muscle visible.
10. Late metamorphosis; the two definitive adductor muscles are now visible. Snapping of the valves cannot be induced by freeing the glochidia from their egg membranes.
11. Juvenile mussels; the foot is now visible, active snapping of the valves and extension of the foot occurs.

[1] No attempt was made to describe fully the various stages of development; they are merely characterized for greater accuracy in determining the duration of developmental stages. Examination was made with a binocular microscope magnifying × 50.

Even juvenile mussels are found within the egg membranes. A stringy transparent gel forms or is secreted about the egg membrane enclosing the developing embryos after they reach the marsupia. It is well formed by the stage at which they begin to turn yellow and partly disappears in late metamorphosis.

Table II. includes observations on twenty typical individuals. More than one hundred are included in the data from which the average time required for the transition between stages is determined.

TABLE II.

STAGES OF DEVELOPMENT OF YOUNG IN MARSUPIA OF *A. imbecillis*.[2]

No. of Mussel.	Date.								
	7/24	7/27	7/31	8/4	8/9	8/15	8/22	8/28	9/1
1	3	4	6	7	8	9	0	0	1
2	3	4	6	7	8	9	0	3	4
3	3	4	5	6–7	8	9	0	1	1–2
4	6	7	8	9	0	0	0	0	1
5	:	:	3	4	6	:	9	0	1
6	:	:	3	4	6	8	9	0	0
7	:	:	7	8	9	0	0	1	3
8	:	:	:	8	9	1	3	7–8	9
9	:	:	:	8–9	0	0	1	4	7
10	:	:	:	5	7	8–9	0	0	3
11	:	:	:	6	7	8–9	0	1	3
12	:	:	:	5	7	8–9	0	0	3
13	:	:	:	1–2	5	7	8–9	0	3
14	:	:	:	1	5	7	9	1	4
15	:	:	:	3	4	6	8–9	0	3
16	:	:	:	4	6–7	7–8	9	0	1
17	:	:	:	1	4	6	9	10–11	0
18	:	:	:	0	0	1	5	8	9
19	:	:	:	0	1	5	7–8	10–11	0
20	:	:	:	6	8	10	0	1	3

Numbers 13, 14, 17, 19 underwent a complete reproductive cycle in from 22 to 27 days during August. The calculation of cycle duration by averaging parts of cycles from all available data gives an average cycle duration of 3 to 4 weeks. This period then provides time for fertilization, segmentation, embryonic development to the glochidial or larval stage, and metamorphosis of the glochidium into the juvenile mussel. In *Anodonta imbecillis* it all occurs in the maternal marsupium, with the possible exception of fertilization. Metamorphosis, which in

[2] 0 in the table indicates empty marsupia during the interval between reproductive cycles. Numbers refer to stages characterized in the text.

other forms occurs in epithelial "cysts" on the gills of fishes, requires in this species only 7–10 days, as compared with a minimum of 12–14 in *Lampsilis anodontoides* and *L. luteola*. Development from the fertilized ovum to the fully formed glochidium may occur in two weeks' time.

The duration of the interval between reproductive cycles varies from 2 or 3 days to 2 (or rarely 3)weeks.

It is realized that more extensive observations would provide material for more accurate determination of the duration of various stages of this cycle and the possible changes in these time relations during the fall and winter months.

However, the data at hand does warrant the conclusion that *Anodonta imbecillis* cannot properly be classed as a long term breeder. Juveniles are discharged during the latter half of August and first half of September and a new reproductive cycle begins. Whether this cycle is repeated throughout the months when this species is listed as gravid is still to be determined.

Discussion.

The possible correlation of the rapid rate of growth and the early attainment of sexual maturity of *Anodonta imbecillis* with the thin "paper shell" has already been mentioned. It is possible that this short reproductive cycle may be correlated with these factors.

No attempt was made to determine whether self or cross fertilization is the normal process in *Anodonta imbecillis*. If self fertilization is the rule, hermaphroditism may have been a contributing factor in the establishment of a shorter reproductive cycle.

Since to date only two species have been reported as developing aparasitically, the idea that they may have depended at one time upon parasitic metamorphosis, but later evolved an ability to develop independently, seems natural. Many investigators have commented on the existence of small hooks at the valve edges as constituting the remains of a former parasitic adaptation. Since parasitism on fishes which make seasonal upstream migrations furnishes an efficient method of distribution (possibly the only method of extensive upstream migration of mussels), a wide distribution of aparasitic forms would lend added weight to

this theory. But if the natural course of evolution of reproduction be followed, parasitism must be considered a secondary development. It is possible therefore that metamorphosis without parasitism is the primitive method and that *Anodonta imbecillis* never has relied upon parasitism for development. It is also possible that the shorter reproductive cycle is the primitive one rather than an adaptation from one similar to that of other species of Unionidae.

SUMMARY.

Anodonta imbecillis cannot be classed with either long or short term breeders as defined by Lefevre and Curtis. In specimens obtained at Fairport, Iowa, a complete reproductive cycle (including metamorphosis from glochidia to juvenile mussels, for reproduction in this species does not depend on parasitism) is completed in from 3 to 4 weeks during the late summer. Another cycle begins after an interval varying from 2 to 3 days to 2 weeks. Gravid *A. imbecillis* have been found in all months but April. It is possible therefore that this short reproductive cycle is repeated throughout the year.

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RESISTANCE OF RHABDITIS TO ACIDS.

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As is well known, *Anguillula aceti* lives in vineger and is highly resistant to acetic acid. Henneberg (1) has shown that this nematode can live in 13.5 per cent. acetic acid. Abbott and Richards have reported that it can live for more than 24 hours in Tellyesnicky's fluid, and for three or four hours in Gilson's fluid.

The writer intended to see in the first place whether other free-living nematodes, which do not come in contact with acids in their natural life, were resistant particularly to acetic acid as well as other acids. For this purpose *Rhabditis elegans* was chosen. In the course of the work it was found that *Rhabditis* is more resistant than the tadpole, *Daphnia*, *Aelosoma*, and *Paramecium* not only to various kinds of acids, but to other toxic substances as well. The cuticle with which nematodes are covered is known to be composed of a very resistant substance; experiments, therefore, were tried to determine whether or not the cuticle is responsible for this resistance, the worms, injured and uninjured, being stained in solutions of neutral red and of methylene blue. This work was begun at the suggestion of Dr. M. H. Jacobs, whom the writer wishes to thank for valuable advice.

Adult hermaphrodites of *Rhabditis elegans* and tadpoles about 10 mm. long and with external gills were used. *R. elegans* is found in decayed matter and is easily cultivated in a peptone solution. Every experiment was repeated at least twice, and 14 to 30 individuals were used for each experiment. The temperature at which experiments were done varied from 21° C. to 26° C.

As shown in Table I., *Rhabditis* is the most resistant to various kinds of acids. It might be noted that it can live about two hours in N/30 acetic acid, while *Daphnia*, the form next in order of resistance, can live only about an hour and a quarter in N/100 acetic acid. From the results of the experiments given in the table we notice, however, that *Rhabditis* is not particularly resistant to acetic acid as compared with other acids.

TABLE I.

AVERAGE TIME IN MINUTES REQUIRED TO CAUSE CESSATION OF ALL MOVEMENTS.

	Rhabditis.	*Paramecium.*	*Aelosoma.*	*Daphnia.*	*Tadpole.*
HCl, N/100	60	<1	<1	23	12
HCl, N/50	17	<1	<1	9	7
Acetic acid, N/10	23	<1	<1	6	—
Butyric acid, N/10	13	<1	<1	—	<1
Salicylic acid, N/100	26	<1	<1	13	—

Is this resistance of *Rhabditis* due to a mechanical reason or to the ability of the animal to neutralize acids? The results of the experiments with toxic substances (Table II.) seem to show that the first is the case, since *Rhabditis* is the most resistant of the five forms studied not only to acids, but to the other toxic substances as well. If this presumption is correct, the cuticle with which *Rhabditis* is covered may be supposed to prevent the penetration of the toxic substances.

TABLE II.

AVERAGE TIME IN MINUTES REQUIRED TO CAUSE CESSATION OF ALL MOVEMENTS.

	Rhabditis.	*Paramecium.*	*Aelosoma.*	*Daphnia.*	*Tadpole.*
NaOH N/40	38	<1	<1	15	2
Ether 5 per cent.	After 10 all revived	<1	<1	After 10 none revived	After 10 none revived
$HgCl_2$ 0.05 per cent.	60	<1	<1	25	5

We notice in these tables that *Rhabditis* is about 2.5 times as resistant to N/40 NaOH, 0.05 per cent. mercuric chloride and N/100 HCl than is *Daphnia*, the next most resistant form. Tadpoles died more quickly in N/20 NaOH than in N/20 HCl. As shown in Table II., tadpoles died in 2 minutes in N/40 NaOH, while they died in about 4 minutes in N/30 HCl. This is probably due to the fact that the acid coagulates the body proteins as it penetrates, while the NaOH dissolves them. Tadpoles, which were placed in the 5 per cent. solution of ether for 5 minutes, revived though they were in a very bad condition. None of the *Rhabdites* revived after placing them 30 minutes in the solution of ether.

In order to study the penetration of an intra-vitam stain, some

Rhabdites were placed in a small quantity of neutral red solution on a slide, and were observed under a microscope. The stain entered through the mouth, and soaked out through the wall of the esophagus. In one worm the stain entered through the anus, but only a small region of the posterior part of the body was stained.

A solution of neutral red, which stained dead worms deeply, stained living worms only faintly; a strong solution, therefore, was used. Staining for 2.5 hours in such a strong solution, the anterior part of the body as faras the second bulb was deeply stained, but in the remaining part the intestine only was colored. The intestine was stained less deeply than the anterior regions of the body, and the stain became progressively lighter toward the posterior end. This seems to show that the stain entered through the mouth, but not through the cuticle nor through the anus. After staining for 20 hours the worms were still alive, and the anterior regions a little beyond the bulb of the esophagus were deeply stained. In the remaining part the intestine only was colored, and its posterior regions were stained less deeply than its anterior regions.

Such stained worms were placed in a weak solution of NaOH, and then the alkali entered through the mouth, and proceeded towards the interior. In about 20 minutes the portion of the body as far as the second bulb became yellow, but the change in color did not go further. Later a rapid diffusion of NaOH from the posterior end anteriorly was observed, and thus the entire body of the worm changed to yellow; the alkali seemed to enter through the anus. In another worm the change in colour proceeded anteriorly starting from the anus.

To show definitely that the cuticle prevents the entrance of the stain and the NaOH, the posterior regions of some worms were cut off. Then the stain entered through the mouth and the cut end. Such worms were, therefore, stained in toto for 4 hours, and at the end of this time they were still alive. Contrary to the case of uninjured worms the change in color carried by NaOH began at both ends, and proceeded faster anteriorly from the cut end than from the mouth. In about one hour and three quarters the change in color has been completed in such injured worms.

As in the case of neutral red, methylene blue entered through the mouth, and in no case penetrated through the cuticle. To stain the entire body of Rhabditis a strong solution was used for the same reason which has been stated in the case of neutral red. After staining 24 hours, some worms were still alive, but others were dead. The anterior part of the living worms as far as the second bulb was deeply stained, but the remaining part was lighter in colour.

Such stained worms were placed in 83 per cent. alcohol. The disappearance of the color began at the anterior end. The worms soon died in the alcohol, and the latter then entered through the anus. As far as the second bulb of the esophagus or a little beyond it, the disappearance of the color was due to the alcohol which entered through the mouth; in the remaining regions to that which entered through the anus. A small region of the anterior part of the intestine still remained stained after one hour in alcohol. In one worm I observed that the posterior division of the hermaphrodite reproductive organs was still stained, while the posterior region of the intestine which is found beside them was already reduced in color.

As in the case of neutral red, in some worms whose posterior ends had been cut off, the stain and alcohol entered through the mouth and the cut end.

Summary.

1. *Anguillula aceti* is highly resistant to acetic acid. To see what is the case in other free-living nematodes *Rhabditis elegans* was used, and it was found that *Rhabditis* is much more resistant not only to various kinds of acids, but to other toxic substances also, as compared with the tadpole, *Daphnia*, *Aelosoma* and *Paramecium*.

2. When animals were stained in neutral red and in methylene blue, no case in which the stain entered through the cuticle was observed.

3. The entrance of NaOH and alcohol through the cuticle also could not be observed.

4. The stains, NaOH and alcohol entered through the mouth, and when the anus and vulva were opened, entered through them as well.

5. In the case of worms whose posterior ends had been cut off these substances in question entered through the cut end, as well as the mouth.

6. The conclusion to be drawn from these results seems to be that the impermeability of the cuticle is responsible for the resistance of *Rhabditis*.

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